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# Inability to Induce Fragile Sites at CTG Repeats in Congenital Myotonic Dystrophy

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**Myotonic dystrophy (DM) is a trinucleotide repeat syndrome which can contain 50 to over 2,000 CTG repeats in affected individuals, but does not express a fragile site. Although one prior study [Jalal et al., Am J Med Genet 46:441–443, 1993] did not find evidence of fragility at 19q13.3 in six individuals affected with DM using induction protocols for folate sensitive fragile sites, other chemicals may induce fragile site expression at this site. In an attempt to induce fragile sites at 19q13.3, blood cultures from four congenital DM cases and four control individuals treated with fluorodeoxyuridine (folate-sensitive rare fragile sites), bromodeoxyurdine (rare and common fragile sites), aphidicolin (common fragile sites), and 5-azacytidine (common fragile sites) were harvested using routine cytogenetic technique. Slides were solid stained and 100 cells were examined for fragile site expression, particularly on F group chromosomes. The latter were photographed prior to destaining and G-banded to verify chromosome and band location of breakage. No culture conditions induced a fragile site at band 19q13.3 at >1% expression in patients with congenital DM. Our results suggest that CTG repeats, even when present in >1,000 copies, may behave differently from other large expansions which are associated with fragile sites. The CTG repeats in DM are not associated with a methylated CpG island, as are folate-sensitive fragile sites, which most likely plays a role in the expression of fragile sites at the trinucleotide repetitive site.** © 1996 Wiley-Liss, Inc.

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## INTRODUCTION

Myotonic dystrophy (DM) is an autosomal dominant disorder characterized by myotonia, progressive muscular weakness and atrophy. DM is divided into three clinical groups: minimally affected or late onset, classical adult onset and congenital form inherited from an affected mother. The disorder has shown the phenomenon of anticipation [Harper, 1989], with earlier age of onset and increasing severity of the disease in successive generations.

The DM gene which contains a CTG trinucleotide repeat in the 3′ untranslated region [Brook et al., 1992] codes for a protein kinase, and has been mapped to chromosome 19 at band q13.3 [Johnson et al., 1990]. Normal individuals have 5–30 copies of the trinucleotide, while affected individuals have 50 to over 2,000 copies [Brook et al., 1992]. The size of the repeat correlates to age of onset and severity of disease [Harley et al., 1993]; individuals with congenital DM have the highest number of repeats [Tsilfidis et al., 1992]. The trinucleotide repeats in affected individuals exhibit meiotic instability [Tsilfidis et al., 1992; Harley et al., 1993], that is, the number of repeats are increased when passed on to offspring, which would explain anticipation seen in families.

Expression of a folate sensitive fragile site at the DM locus was recently tested by Jalal et al. [1993] in six affected individuals, including one with congenital DM. They used three different folate sensitive systems: reduced folic acid, 5-fluorodeoxyuridine (FdU), and high thymidine content, all which affect the de novo thymidylic acid pathway. No evidence of fragility at 19q13.3 suggested that the amplified region is not late replicating or methylated [Jalal et al., 1993]. The large number of repeats in DM, particularly in congenital cases, would suggest that the area could be prone to breakage. Although a folate-deficient system may not induce fragile sites at the DM locus since the repeat is CTG rather than CGG or GCC, it may be inducible by other chemicals.

We therefore examined fragile site expression at 19q13.3 in congenital cases of DM using different fragile site induction systems: FdU (folate sensitive rare fragile sites), aphidicolin (APC; common fragile sites), bromodeoxyuridine (BrdU; rare and common fragile sites), and 5-azacytidine (5-azac; common fragile sites). These studies will determine if the large expansion (>200 repeats) is enough to cause breakage, or if breakage may be related to methylation or late DNA replication. This information will help further our understanding of this amplified repeat syndrome that is to date different from the other syndromes that have been identified.

## MATERIALS AND METHODS

Genomic DNA was prepared for Southern blot analysis using the technique described by Buxton et al. [1992]. DNA was digested using EcoRI and Bam HI. Digested DNA was run overnight on an 0.8% agarose gel and probed with p5B1.4 probe.

Peripheral blood samples from four patients with DM and four control individuals were cultured in 5 ml medium 199 for 72 hours. Six cultures per patient consisted of 0.2 $\mu$M APC during the last 24 hours [Hecht et al., 1988], 0.025 $\mu$M FdU during the last 24 hours [Wenger et al., 1987], 200 $\mu$g BrdU during the last 24 hours [rare fragile sites; Hecht et al., 1988], 10 ug 5-azac during the last 7 hours [Sutherland et al., 1985], 250 ug BrdU during the last 6 hours [common fragile sites; Hecht et al., 1985], and one untreated culture. Cells were harvested using routine cytogenetic technique. At least one slide from each individual was G-banded and cells were karyotyped. Non-banded chromosome preparations were stained with Giemsa and 100 cells were examined for fragile sites. Gaps and breaks on all chromosomes were documented. Cells with gaps or breaks on F group chromosomes were destained and G-banded for identification of chromosome 19 and band location of break. Expression at 4% or higher would indicate presence of a fragile site at 19q13.3.

## RESULTS

The CTG repeat size determined by Southern blot analysis ranged from 1,050 to 1,800 in the patients with congenital DM. The midpoint of the heterogeneous smear produced by the Southern blot was used to estimate the CTG copy number. Generally, all DM patient samples had a range of repeat sizes ±200 repeats from the midpoint estimation. Karyotypes for all patients and controls were normal. Chromosome gaps and breaks were 10% or higher in cultures scored under various medium conditions with the exception of APC treatment for control 3 (Table I). Breakage at 19q13.3 was seen rarely (1%) in one or more cultures for each of the patients with congenital DM (Table II). 5-azac induced stretching of C band heterochromatic regions for chromosomes 1, 9, and 16 [Sutherland et al., 1985], while APC and BrdU induced the most frequently reported fragile sites [Hecht et al., 1988]. Induction of the rare fragile sites could not be verified; however, the FdU system is used clinically for identifying fragile X positive cases [Wenger et al., 1987].

## DISCUSSION

Dominantly inherited diseases with CAG trinucleotide repeats include spinobulbar muscular atrophy [LaSpada et al., 1991], Huntington disease [Gusella et al., 1993], and dentatorubral-pallidoluysian atrophy [Koide et al., 1994; Nagafuchi et al., 1994], usually with less than 100 CAG repeats. The CAG repeats are in the translated region and are found in the protein, suggesting a gain of function for the disease state. CGG and GCC repeats have been identified in fragile X syndrome [Kremer et al., 1991] and fragile X E mental retardation syndrome [Knight et al., 1993], respectively. These syndromes have similarities with DM in that the number of repeats in affected individuals can be quite high (>200) and the repeats are in an untranslated region. The fragile X syndromes are also associated with rare folate sensitive fragile site expression at the location of the expanded trinucleotide repeats (Xq27.3, FRAXA and Xq28, FRAXE) which is induced by blocking de novo synthesis of thymidylic acid [Glover, 1981]. Fragile sites FRAXF [Ritchie et al., 1994] and FRA16A [Nancarrow et al., 1994] are also similar to FRAXA and FRAXE except that they are not associated with a genetic disorder. FRA11B, also having similar molecular composition to the other folate-sensitive fragile sites, is associated with a chromosome deletion, Jacobsen syndrome [Jones et al., 1995].

TABLE I. Chromosome Breakage Among 100 Cells*

| | Control | FdU | BrdU 6 hr | BrdU 24 hr | APC | 5-AZA |
|---|---|---|---|---|---|---|
| Controls | | | | | | |
| 1 | 31 | — | 55 | 99 | 20 | 31 |
| 2 | 10 | 27 | 60 | 25 | 16 | 34 |
| 3 | 11 | 11 | 14 (43) | 16 | 2 (50) | 38 |
| 4 | — | — | 50 | — | — | 20 |
| Patients | | | | | | |
| 1 | 14 | 14 | 54 | — | — | 15 |
| 2 | 11 | 19 | 13 | 22 | 16 | 57 |
| 3 | 22 | 35 | 43 | 82 | 36 | 44 |
| 4 | 13 | 16 | 67 | 43 | 15 | — |

*Individual chromosome gaps and breaks were scored among 100 cells for each culture condition unless noted in parentheses. Dashes represent no data from culture.

TABLE II. Chromosome Expression of Fragile Sites at 19q13.3*

| | Control | FdU | BrdU 6 hr | BrdU 24 hr | APC | 5-AZA |
|---|---|---|---|---|---|---|
| Control | | | | | | |
| 1 | 0 | — | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | — | — | — | 0 | — | 0 |
| Patient | | | | | | |
| 1 | 1 | 0 | — | 0 | — | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 1 |
| 3 | 1 | 1 | 0 | 1 | 0 | 0 |
| 4 | 0 | 0 | 1 | 0 | 0 | — |

*Breakage on F group chromosomes were G-banded for identification of band location. Data represents breakage among 100 cells.

The above five folate-sensitive fragile sites are associated with methylation at a nearby CpG island when the number of trinucleotide repeats exceeds 200 [Bell et al., 1991; Knight et al., 1993], which at least for fragile X syndrome results in the absence of mRNA [Pieretti et al., 1991]. Gene inactivation has been associated with late or delayed DNA replication, which has been demonstrated in fragile X syndrome both cytogenetically [Yu et al., 1990; Webb, 1992] as well as molecularly [Hansen et al., 1993]. However, methylation has not been found associated with the DM gene [Shaw et al., 1993], and imprinting is not involved in allelic expression [Jansen et al., 1993].

Our results suggest that CTG repeats behave differently from other large expansions. This could be related to several factors: 1) The nucleotides in the repeats are different. While CGG and GCC repeats are folate-sensitive, CTG repeats did not result in increased breakage for any of the chemicals tested, which included all but distamycin A for induction of fragile sites [Sutherland and Ledbetter, 1989]. It may be that different medium conditions are necessary to induce fragile sites at CTG repeats, or that they are resistant to breakage. 2) The location of the repeats are different. For DM the repeats are in the 3′ untranslated region while for FRAXA they are in the 5′ untranslated region. The position in the gene may in some way influence the stability or fragility of the area. 3) DM is not associated with a methylated CpG island while the other folate sensitive fragile sites are. The protein kinase gene is transcribed in patients with DM [Fu et al., 1993; Sabouri et al., 1993], while in the case of FRAXA, methylation prevents transcription of the FMR1 gene [Pieretti et al., 1991]. Absence of methylation may be the most likely explanation for lack of fragility at 19q13.3 regardless of the size of the CTG repeat area. Methylation associated with the CGG repeat in FRAXA may affect the structural orientation of DNA [Eberhart and Warren, 1993], or may cause a delay DNA synthesis [Laird et al., 1987], either of which may make the repetitive region prone to breakage.

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